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# Acute exercise does not induce an acute phase response (APR) in Standardbred trotters

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## Abstract

The purpose of the study was to investigate whether acute strenuous exercise (1600- to 2500-m race) would elicit an acute phase response (APR) in Standardbred trotters. Blood levels of several inflammatory markers [serum amyloid A (SAA), haptoglobin, fibrinogen, white blood cell count (WBC), and iron], muscle enzymes [creatinine kinase (CK) and aspartate transaminase (AST)], and hemoglobin were assessed in 58 Standardbred trotters before and after racing. Hemoglobin levels increased and iron levels decreased 12 to 14 h after racing and haptoglobin concentrations, white blood cell counts, and iron levels were decreased 2 and/or 7 d after racing. Concentrations of CK, AST, SAA, and fibrinogen were unaltered in response to racing.

Acute strenuous exercise did not elicit an acute phase reaction. The observed acute increase in hemoglobin levels and decreases in haptoglobin and iron levels may have been caused by exercise-induced hemolysis, which indicates that horses might experience a condition similar to athlete's anemia in humans. The pathogenesis and clinical implications of the hematological and blood-biochemical changes elicited by acute exercise in Standardbred trotters in the present study warrant further investigation.

## Résumé

L'objectif de la présente étude est d'investiguer l'effet de l'exercice intensif (1600–2500 m) sur l'apparition d'une phase inflammatoire aiguë chez des trotteurs. Les taux sanguins de différents marqueurs inflammatoires [serum amyloid A (SAA), haptoglobine, fibrinogène, globules blanc (WBC) et fer], d'enzymes musculaires [creatinine kinase (CK) et aspartate transaminase (AST)] et d'hémoglobine ont été évalués dans 58 trotteurs avant et après la course. Respectivement une augmentation et diminution des taux d'hémoglobine et de fer furent présente 12–14 heures suivant la course. Une réduction des taux d'haptoglobine, de globules blanc et de fer était présente 2 et/ou 7 jours après la course. Aucun changement dans les concentrations de CK, AST, SAA et fibrinogène n'a pu être démontré en relation avec la course.

L'exercice intense réalisé lors de cette étude n'a pu induire de phase inflammatoire aiguë. L'augmentation d'hémoglobine et la diminution d'haptoglobine et de fer peuvent être causées par une hémolyse induite par l'exercice, indiquant qu'une condition similaire à l'anémie de l'athlète chez l'homme existe chez le cheval. La pathogénèse et l'implication clinique des modifications hématologiques et biochimiques liées à un exercice intense chez les trotteurs dans la présente étude justifient de plus amples investigations.

(Traduit par Docteur Denis Verwilghen)

## Introduction

The acute phase response (APR) is the rapid and non-specific response elicited by all sorts of inflammatory stimuli, such as infection, trauma, cancer, and ischemia. It is characterized by release of signal molecules from injured cells and tissues; these molecules trigger synthesis of pro-inflammatory cytokines that will induce hepatic synthesis of acute phase proteins (APPs). The APPs are released into the blood stream, where they can be measured in increased concentrations (1). In the horse, several proteins have acute phase properties, e.g., serum amyloid A (SAA), fibrinogen, and haptoglobin. In response to inflammatory stimuli, blood concentrations of fibrinogen and haptoglobin will increase modestly (up to 10 times healthy values), while SAA concentrations may increase several 100- or 1000-fold (2). Along with levels of APPs, levels of other parameters such as white blood cell counts (WBC) and iron concentrations will

change during the inflammatory response. While WBC will increase during inflammation, serum iron concentrations will decrease (3,4).

It has been shown that strenuous exercise elicits a short-lived APR in humans and horses, with increases in WBC and serum concentrations of APPs. Most of these studies have investigated the APR in endurance sports such as 120- to 160-km endurance races in horses (5,6) and marathon, ultramarathon, and triathlon competitions in humans (7). There are, however, studies in humans, dogs, and horses that have investigated the effect of acute exercise of a shorter duration at high intensity. Two studies of trained human amateur runners (8,9) investigated the response to shorter running distances (5 and 15 to 21 km), as did a study in sled dogs (10) performing moderate-duration, high-intensity exercise (racing 16 miles each of 2 days). These studies showed that shorter running distances also caused an APR with small and short-lived increases in WBC and serum concentrations of C-reactive protein, the major human and canine

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APP. In contrast, a recent study of horses participating in limited distance endurance rides (34 or 60 km) showed no post-race change in APPs (6). The APR after acute, short-duration exercise (sprinting) has been investigated to a limited degree in horses. One study of Thoroughbreds showed no SAA response 1 h after a 1600-m race (11). No studies are available on the effects of racing on the APR of Standardbred trotters.

The APR to exercise seems to be related to running distance (9) and degree of muscle injury, as measured by serum concentrations of creatinine kinase (CK) (12,13). Training has been shown to cause a reduction in the post-run APR in humans (14), thus suggesting that an improved physical condition protects against the exercise-induced APR. In contrast, a previous study in Thoroughbreds demonstrated increased fibrinogen and haptoglobin concentrations during the last weeks of an 80-d training program. It was suggested that this could be an effect of both the adjustments to training and subclinical disease becoming manifest over time (15).

The hypothesis of this study was therefore that acute, strenuous exercise (1600 to 2500 m-race) induces an APR in Standardbred trotters. Assessing levels of acute phase reactants could potentially yield information about individual fitness and the possible need for a prolonged rest after racing.

## Materials and methods

Blood samples were obtained from 58 Standardbred trotters [22 stallions, 13 mares, 23 geldings; mean age 4.8 y (from 3 to 11 y)]. Pre-race samples (day -1) were collected from all horses. Samples were collected 12 to 14 h after racing (group A,  $n = 26$ ) or 2 and 7 d after racing (group B,  $n = 32$ ). The horses were all fit racehorses in professional training that raced at different racetracks in Denmark and Sweden. Racing distances were 1600 m ( $n = 1$ ), 1640 m ( $n = 11$ ), 1700 m ( $n = 5$ ), 1800 m ( $n = 2$ ), 1900 m ( $n = 7$ ), 2000 m ( $n = 1$ ), 2020 m ( $n = 1$ ), 2100 m ( $n = 5$ ), 2140 m ( $n = 18$ ), and 2500 m ( $n = 6$ ), all of which are considered sprinting distances. Racing distance was not recorded for 1 horse. Information about racing speed (kilometer time, i.e., time in seconds when trotting a 1-km distance) and finishing position was recorded for each horse.

On the day of blood collection, each horse underwent a general clinical examination. Blood was collected in tubes (Vacutainer Systems; Becton Dickinson, Meylan, France) containing sodium-ethylene diamine triacetic acid (EDTA) for determining WBC and hemoglobin, tubes containing sodium citrate for determining plasma fibrinogen, and tubes with no additive for preparing serum samples for analyzing concentrations of SAA, iron, CK (only measured in group A), aspartate transaminase (AST) (only measured in group A), and haptoglobin (only measured in group B). Citrated plasma was prepared by centrifugation at  $2000 \times g$  for 10 min. Serum was prepared by letting blood samples coagulate for approximately 6 h before centrifugation at  $2000 \times g$  for 10 min. Analyses were carried out immediately (WBC, hemoglobin) or serum/plasma was stored for up to 4 wk at  $-20^\circ\text{C}$  before analyses were carried out (CK, AST, SAA, haptoglobin, fibrinogen, and iron).

Serum CK and AST were determined spectrophotometrically [by CK-catalyzed N-acetylcysteine-activated reaction with adenosine diphosphate (ADP) and, through several steps, subsequent

**Table 1. Levels of creatinine kinase (CK), aspartate transaminase, acute phase proteins, iron, hemoglobin, and white blood cell count in 26 Standardbred trotters before (day -1) and after (12 to 14 h) racing 1640 or 2140 m. Levels are shown as mean  $\pm$  standard deviation (range). Within each row, different superscript letters signify statistical difference ( $P < 0.05$ ) between sampling times**

	Day -1	14 h
Creatinine kinase (U/L)	316.1 $\pm$ 368.3 <sup>a</sup> (157 to 2035*)	262.2 $\pm$ 79.84 <sup>a</sup> (166 to 454)
Aspartate transaminase (U/L)	434.8 $\pm$ 188.4 <sup>a</sup> (172.0 to 1021)	465.6 $\pm$ 156.5 <sup>a</sup> (170.0 to 825.0)
Serum amyloid A (mg/L)	1.6 $\pm$ 0.23 <sup>a</sup> (1.1 to 2.0)	1.6 $\pm$ 0.36 <sup>a</sup> (1.2 to 2.8)
Fibrinogen (g/L)	3.87 $\pm$ 1.47 <sup>a</sup> (1.98 to 5.73)	3.54 $\pm$ 1.13 <sup>a</sup> (1.89 to 6.01)
Iron ( $\mu\text{mol/L}$ )	29.09 $\pm$ 8.93 <sup>a</sup> (15.48 to 59.47)	25.47 $\pm$ 6.17 <sup>b</sup> (15.44 to 37.36)
Hemoglobin (g/L)	138.7 $\pm$ 10.12 <sup>a</sup> (118.0 to 157.0)	150.6 $\pm$ 11.77 <sup>b</sup> (130.0 to 170.0)
White blood cell count ( $\times 10^9/\text{L}$ )	7.27 $\pm$ 1.24 <sup>a</sup> (5.6 to 9.9)	7.86 $\pm$ 1.31 <sup>a</sup> (5.6 to 10.0)

\* One horse had elevated CK before racing, but in a control sample obtained 1 h after racing, levels were 600 U/L (data not shown) and the measured value may be an error or result from contamination of the phlebotomy needle with tissue. As no reason for excluding this outlier was identified, it was retained in the analyses to avoid bias.

reduction of nicotinamide-adenine dinucleotide phosphate (NADP) to nicotinamide-adenine dinucleotide phosphate plus hydrogen (NADPH), and by AST-catalyzed L-aspartate and 2-oxoglutarate reaction with subsequent maleate dehydrogenase-catalyzed NADH oxidation indicator reaction] according to International Federation of Clinical Chemistry guidelines in an ADVIA 1800 Chemistry System (Siemens Health Care Diagnostics, Deerfield, Illinois, USA). Serum SAA concentrations were determined by a previously validated immunoturbidometric method (LZ test SAA; EIKEN Chemical, Tokyo, Japan) (16). Haptoglobin was assessed by colorimetric spectrophotometry (Phase Range Haptoglobin Assay; Tridelta Development, Maynooth, Ireland) on an automated ADVIA 1800 Chemistry System (Siemens Health Care Diagnostics) as described in a previous study (17). Fibrinogen concentrations were determined by the Clauss method in an automated coagulometric analyzer (ACL 9000; Instrumentation Laboratory, Barcelona, Spain). Serum iron concentrations were determined by colorimetric spectrophotometry (ADVIA 1650; Bayer A/S, Lyngby, Denmark).

Different analysis techniques were used for the 2 groups of horses to determine WBC and hemoglobin concentrations. In group A, hematological analyses were done using an ADVIA 2120 analyzer (Siemens Healthcare Diagnostics) with equine settings. In group B, 2 handheld analyzers (HemoCue Hb 201+ and HemoCue WBC; HemoCue, Vedbæk, Denmark) were used according to the manufacturer's instructions.



**Table II. Levels of acute phase proteins, iron, hemoglobin, and white blood cell count in 32 Standardbred trotters before (day -1) and after (day 2 and 7) racing 1600 to 2500 m. Levels are shown as mean  $\pm$  standard deviation (range). Within each row, different superscript letters signify statistical difference ( $P < 0.05$ ) between sampling times**

	Day -1	Day 2	Day 7
Serum amyloid A (mg/L)	10.4 $\pm$ 42.2 <sup>a</sup> (below DL-214*)	0.8 $\pm$ 4.2 <sup>a</sup> (below DL-25.1)	Below DL <sup>a</sup> (all below DL)
Haptoglobin (g/L)	1.91 $\pm$ 0.25 <sup>a</sup> (0.70 to 1.59)	1.11 $\pm$ 0.29 <sup>b</sup> (0.52 to 1.55)	1.15 $\pm$ 0.24 <sup>a,b</sup> (0.73 to 1.59)
Fibrinogen (g/L)	3.1 $\pm$ 0.4 <sup>a</sup> (2.2 to 4.0)	3.1 $\pm$ 0.4 <sup>a</sup> (2.3 to 3.9)	3.0 $\pm$ 0.4 <sup>a</sup> (2.3 to 4.2)
Iron ( $\mu$ mol/L)	31.98 $\pm$ 7.8 <sup>a</sup> (18.81 to 52.13)	31.88 $\pm$ 8.2 <sup>a</sup> (16.98 to 54.38)	28.35 $\pm$ 9.2 <sup>b</sup> (16.29 to 60.60)
Hemoglobin (g/L)	149.4 $\pm$ 19.17 <sup>a</sup> (79.0 to 193.0)	150.6 $\pm$ 23.77 <sup>a</sup> (96.0 to 214.0)	148.4 $\pm$ 15.59 <sup>a</sup> (98.0 to 175.0)
White blood cell count ( $\times 10^9$ /L)	8.8 $\pm$ 2.2 <sup>a</sup> (5.2 to 14.5)	8.3 $\pm$ 1.4 <sup>a,b</sup> (5.8 to 10.7)	7.7 $\pm$ 1.5 <sup>b</sup> (5.7 to 11.2)

DL — detection limit of the assay (corresponding to 0.5 mg/L SAA).

\* It was not clear why 1 horse had mildly elevated SAA before racing, as the horse was clinically healthy and SAA levels were low in the subsequent sample. As no reason for excluding this outlier was identified, it was retained in the analyses to avoid bias.

All statistical analyses were carried out using Prism 4.02 (GraphPad, San Diego, California, USA). Normal distribution of data was assessed by D'Agostino and Pearson omnibus normality test and all parameters, except SAA, were found to be normally distributed. Changes over time in WBC and serum levels of CK, AST, SAA, haptoglobin, fibrinogen, and iron within each group were evaluated by paired *t*-test (group A) or repeated measurements analysis of variance (ANOVA) with pairwise comparisons by Bonferroni test for significant effects (group B). Due to the different laboratory analyses used for determining some of the measured parameters in the 2 groups of horses, levels could not be compared between the 2 groups. The correlation between blood parameters and kilometer time was evaluated by linear regression. Differences in blood parameters between racing distances were evaluated by Student's *t*-test (group A) or one-way ANOVA and pairwise comparisons (Bonferroni) for significant effects (group B). A 5% significance level was used.

## Results

All horses remained clinically healthy throughout the study, except for 1 horse that was excluded from the study as it developed a hoof abscess. Five horses galloped during the race and their kilometer times were thus not included in further analyses.

Analyses showed that, in group A, iron decreased significantly ( $P < 0.05$ ) and hemoglobin increased significantly ( $P < 0.05$ ), while CK, AST, fibrinogen, SAA, and WBC remained unchanged (Table I). In group B, haptoglobin, iron, and WBC decreased significantly ( $P < 0.01$ ,  $P < 0.05$ , and  $P < 0.01$ , respectively) (Table II), while SAA, fibrinogen, and hemoglobin concentrations remained unchanged throughout the study period (Table II).

Racing distance and racing speed [kilometer time; average and standard deviation (SD) 76.2  $\pm$  2.2, range 72.0 to 84.5 s/km] were generally not related to magnitude of change in levels of the blood parameters, but in group B, WBC levels decreased to a greater magnitude in horses racing 2500 m than in horses racing shorter distances ( $P < 0.05$ ).

## Discussion

Acute, high-intensity exercise caused changes in the levels of iron, hemoglobin, haptoglobin, and WBC in the blood of the horses. The results of the present study did not, however, support the hypothesis that exercise induces an inflammatory response. Levels of the 4 positive acute phase parameters remained unchanged (SAA and fibrinogen) or decreased (haptoglobin, WBC) after racing. Serum amyloid A concentrations will increase several 100-fold even after mild inflammatory stimuli such as minor arthroscopic procedures (18), foaling (19), or vaccination (20). Even minor injuries to the horses during racing, such as muscle damage, that cause a subsequent APR would thus likely have resulted in increased serum SAA concentrations. The horses in the present study did not seem to suffer muscle damage, as serum levels of CK and AST were not significantly elevated after racing. This is in contrast to horses performing endurance rides of 120 or 160 km, which had elevated serum CK and SAA levels after the race (5,6). Two studies on the effects of training on CK and APPs in Thoroughbreds and Standardbreds showed increases in CK and fibrinogen and haptoglobin over the course of an 80-d training program (15,21). These differences are very likely related to differences in duration of exercise and type of exertion between studies.



Timing of sampling is important in order not to miss a potential increase in SAA and other APPs. Previous studies have shown that serum concentrations of SAA will increase within 8 to 24 h of an inflammatory stimulus, while fibrinogen and haptoglobin responses develop more slowly (18,20,22). No significant change in SAA levels were detected in Standardbreds 1 h after completing a standardized 1600-m race (11), but due to the kinetics of the SAA response, increases in SAA concentrations would not be expected with this short interval between stimulus and sampling. In contrast, the sampling intervals in the current study (12 to 14 h, 2 and 7 d after the race) were long enough to allow SAA, fibrinogen, and haptoglobin responses to develop, if present (2). Therefore, the lack of APP response cannot be ascribed to the choice of sampling times.

The WBC decreased 7 d after racing. The pathogenesis of this decrease is not clear. Taking into account the kinetics of leukocyte response (when fast decreases in leukocyte numbers occur only in response to severe and peracute inflammation such as sepsis), it is not likely that the small, yet statistically significant decrease on day 7 is the result of race-related inflammation. Leukocyte numbers, populations, and functions may change in response to acute exercise (23). Increases in leukocyte numbers are usually observed within hours of acute exercise and the significance and interpretation of the late and small decrease in WBC observed in the present study are thus not clear (23,24). The consequences, if any, of such changes in terms of performance and health of the horses are currently unknown, but in humans, heavy training has been linked to immunosuppression and increased incidence of upper airway infections (23). Further studies are needed to determine whether changes in leukocyte numbers are a consistent feature of acute strenuous exercise in horses and to investigate whether the observed small changes have any clinical significance.

The measurements of the 4 positive acute phase parameters thus did not support the hypothesis of a racing-induced inflammatory response. In contrast, the decrease in serum iron levels observed in both groups of horses might reflect an exercise-induced inflammatory response. Iron is a very sensitive marker of inflammation with a fast kinetic. It will decrease within hours after an inflammatory insult and return to baseline levels 1 to 2 d after inflammation has waned (18,20). Inflammatory cytokines such as interleukin-1 $\beta$ , interleukin-6, and tumor necrosis factor  $\alpha$  are released and cause hepatic upregulation of the hormone hepcidin during inflammation (4) as well as during exercise (25). This has a negative influence on iron transportation and absorption and, as such, exercise-induced inflammation might potentially lead to a drop in serum iron concentrations similar to that demonstrated in other inflammatory conditions in horses (20). In the absence of other inflammatory signs, however, non-inflammatory causes of decreased serum iron concentrations are more likely in the present study. Hemolysis and/or volume expansion have been suggested as potential causes of decreased serum iron in horses undergoing a 3-wk training program (26). In humans, many exercise-related factors can affect serum iron concentrations, e.g., loss of iron in sweat (27), increased erythrocyte destruction with subsequent hemoglobinuria (28), and gastrointestinal bleeding (29). It was not determined whether one or more of these mechanisms contributed to iron loss in the horses involved in the present study.

The increase in blood hemoglobin levels demonstrated in group A 12 to 14 h after racing and the decrease in serum haptoglobin concentration demonstrated in group B 2 days after racing may indicate that subclinical hemolysis occurred in response to the exercise, which is similar to findings in numerous studies in humans (28,30–34). Haptoglobin associates strongly with hemoglobin released from red blood cells during hemolysis. Decreased serum haptoglobin concentrations have thus been demonstrated in horses after intravascular hemolysis (35), and 2 studies have shown that hemoglobin levels increase and haptoglobin levels decrease in Thoroughbreds after individual training sessions or over the course of a 3-wk training program (26,36). As hemoglobin in the present study was total hemoglobin, not plasma hemoglobin, increases in numbers of red blood cells, e.g., in response to splenic contraction, may also have affected the results.

Hemolysis and changes in iron metabolism such as those demonstrated in the present study are thought to be the cause of athlete's anemia. This condition, which is characterized by persistently low levels of blood hemoglobin, low hematocrit, and low ferritin concentrations, is well-described in humans, particularly in athletes practicing endurance sports. It has been suggested that this condition causes suboptimal performance or even fatigue. Several factors may contribute to its development in humans, including hemolysis caused by the foot striking against hard ground, gastrointestinal bleeding, hematuria, sweating, and menstruation in females (37). Additional studies are needed with more detailed analyses of factors involved in erythrocyte turnover and iron metabolism to determine whether anemia occurs in equine athletes. It is not clear if and how changes in erythrocyte-related parameters, haptoglobin levels, and iron status affect the equine athlete clinically or in terms of performance.

In conclusion, the acute exercise performed in the present study did not elicit any kind of inflammatory response with changes in levels of acute phase parameters. This is in contrast to studies in endurance horses after 120- to 160-km rides (5,6), and is most likely explained by the short racing distance and different type of exercise performed by the Standardbreds (1600- to 2500-m racing). For racehorses, races of this distance are considered sprinting and the effect of sprinting on the APR has not previously been investigated in Standardbred trotters. In contrast, blood-biochemical changes were demonstrated that were consistent with acute hemolysis (increased hemoglobin and decreased haptoglobin and iron), which might suggest that even short bouts of strenuous exercise by the horses can lead to changes similar to those observed in human athletes and horses after acute short or longer term exercise (6,30,36). It needs to be determined whether these changes affect the performance and/or health of the horses.

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